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Visible Light-Driven H_2 Production by Hydrogenases Attached to Dye-Sensitized TiO_2 Nanoparticles

Erwin Reisner,[†] Daniel J. Powell,[†] Christine Cavazza,[‡] Juan C. Fontecilla-Camps,[‡] and Fraser A. Armstrong*,[†]

Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford, OX1 3QR, United Kingdom, and Laboratoire de Crystallographie et Crystallogènese des Protéines, Institut de Biologie Structurale, J.P. Ebel, CEA, CNRS, Université Joseph Fourier, 41, rue J. Horrowitz, 38027 Grenoble Cedex 1, France

Received September 17, 2009; E-mail: fraser.armstrong@chem.ox.ac.uk

Abstract: A study of hybrid, enzyme-modified nanoparticles able to produce H_2 using visible light as the energy source has been carried out to establish per-site performance standards for H_2 production catalysts able to operate under ambient conditions. The [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum* (*Db* [NiFeSe]-H) is identified as a particularly proficient catalyst. The optimized system consisting of *Db* [NiFeSe]-H attached to Ru dye-sensitized TiO_2, with triethanolamine as a sacrificial electron donor, produces H_2 at a turnover frequency of approximately 50 (mol H_2) s^{-1} (mol total hydrogenase)$^{-1}$ at pH 7 and 25 °C, even under the typical solar irradiation of a northern European sky. The system shows high electrocatalytic stability not only under anaerobic conditions but also after prolonged exposure to air, thus making it sufficiently robust for benchtop applications.

Introduction

Production of dihydrogen (H_2) by water splitting (electrochemical, photochemical, or thermal) offers an obvious way to capture solar energy and thus provide "greener" energy for the future.[1−4] Not only is H_2 a fuel itself, but also it can be processed to produce other fuels (Fischer−Tropsch reactions), and it is an essential material for the organic chemical industry and for producing fertilizers by the Haber−Bosch process. Catalysts are a crucial issue for H_2 production, and there is intense interest in finding alternatives to noble metals (notably Pt) that are capable of converting water into H_2 close to the thermodynamic potential.[5] The cost and limited availability of Pt, coupled with its nonselectivity and poisoning by environmental pollutants, are serious restrictions to future generation of H_2 by renewable means. Industrial scale water electrolysis is commonly performed at 80−85 °C with strong alkali solutions and Fe/Ni electrodes, but large overpotentials are required, so the efficiency is poor.[6] On the closely related photochemical front, there is great interest in coordination complexes able to perform all of the tasks required, photon capture and catalysis of O_2 and H_2 production, all integrated within well-defined structures;[7,8] although, to simplify the task, investigations have focused on understanding and improving the redox half-reactions separately of each other.[9] Although O_2 production is generally acknowledged as the major kinetic obstacle to photochemical or electrochemical water splitting, there is still no chemical catalyst able to produce H_2 with the minimal overpotential displayed by Pt.

As a biological alternative,[10−12] enzymes known as hydrogenases catalyze the reduction of protons into H_2 at active sites composed of iron- or nickel/iron complexes: importantly, the electrochemical reaction is reversible, as with Pt. Determination of the structures of hydrogenases from several organisms paired with detailed spectroscopic and electrochemical investigations have considerably improved our understanding of these enzymes in recent years.[13−15] Electrochemical studies have established that hydrogenases can produce or oxidize H_2 with just a minimal overpotential and achieve turnover frequencies of up to tens of thousands per second in neutral aqueous solution at room

[†] University of Oxford.
[‡] Université Joseph Fourier.

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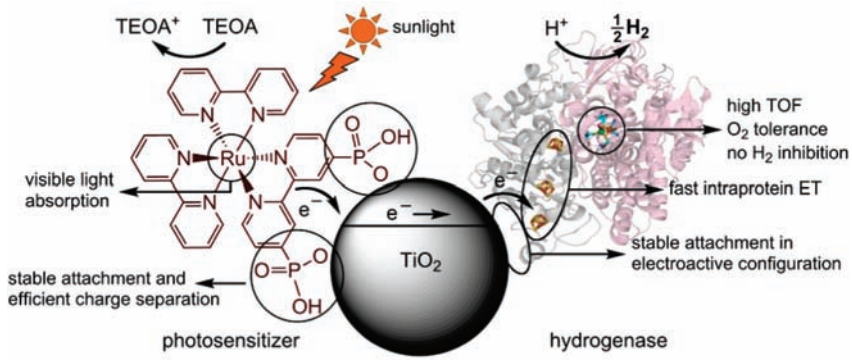

Figure 1. Cartoon representation of a hybrid (enzyme−TiO_2) nanoparticle system showing aspects that are desirable for efficient and practical H_2 production from sunlight. The system is shown with a hydrogenase (*Db* [NiFeSe]-H) as catalyst and the complex (RuP) that proved to be the most suitable photosensitizer.

temperature.[16,17] This activity is often exhibited in the presence of inhibitors such as CO and H_2S, conditions under which nonbiological H_2 production catalysts are poisoned, and even O_2, which would be a byproduct in complete water splitting photosystems. Enzymes therefore constitute important performance targets for assessing and developing future catalysts.[17]

The ability to attach hydrogenases with high electrocatalytic activity to a graphite electrode surface has been exploited recently in several inspiring demonstrations:[18] (i) an O_2-tolerant, H_2 oxidizing [NiFe]-hydrogenase from *Ralstonia metallidurans* adsorbed on a pyrolytic graphite edge (PGE) electrode was employed as the anode in an enzyme-fuel cell able to operate in air containing just 3% H_2;[19] (ii) *Clostridium acetobutylicum* [FeFe]-hydrogenase Hyd-A (*Ca* [FeFe]-H) adsorbed on a PGE electrode was shown to produce H_2 with simultaneous oxidation of NADH when incorporated into a photoelectrochemical enzyme-fuel cell;[20] and (iii) conducting graphite microparticles coated with a [NiFe]-hydrogenase and a carbon monoxide dehydrogenase (CODH, another Ni- and Fe-containing enzyme) were demonstrated to catalyze the industrially important water-gas-shift reaction under ambient conditions.[21]

We recently reported on the stable electrochemistry of a [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum* (*Db* [NiFeSe]-H) on a TiO_2 electrode. This observation led to a prototype solar H_2 production system consisting of this hydrogenase and a synthetic ruthenium photosensitizer, co-attached to colloidal TiO_2 nanoparticles.[22] In view of the significance of this experiment for demonstrating the possibilities for solar-H_2 production, it was necessary to determine the importance of choosing a particular enzyme and photosensitizer and to identify operational conditions leading to optimal performance. We now describe the results of extensive investigations. Figure 1 outlines the desirable features for an efficient and practical system: it includes the hydrogenase and the photosensitizer "RuP" that we found to function most proficiently among the options examined.

Experimental Section

General Considerations and Materials. All starting reagents were obtained from commercial suppliers; they were of the highest available purity and used as received unless otherwise noted. The compounds $[Ru(bpy)_2(H_4dpbpy)]Br_2$ (**1** = RuP, bpy = 2,2′-bipyridine, H_4dpbpy = 2,2′-bipyridine-4,4′-diylbis(phosphonic acid)),[23] $[Ru(bpy)_2(H_2dcbpy)]Cl_2$ (**2**, H_2dcbpy = 2,2′-bipyridine-4,4′-dicarboxylic acid),[24] *cis*-$[Ru(NCS)_2(H_2dcbpy)]$ (**3** = N3),[25] $[PtCl_2(H_2dcbpy)]$ (**4**),[26] and $[Pt(bdt)(H_2dcbpy)]$ (**5**, bdt = 1,2-benzenedithiolate)[27] were prepared as reported previously, and their composition and purity were confirmed by ^{1}H NMR, IR, and UV−vis spectroscopy. Purified water (Millipore: 18 MΩ cm) was buffered with triethanolamine (TEOA, 25 mM), which also acted as sacrificial electron donor, and titrated with dilute HCl to the desired pH at the experimental temperature. Sodium chloride (0.10 M) was added to the TEOA buffer as supporting electrolyte for the electrochemical measurements. The nanoparticles (AEROXIDE TiO_2 P25 particles from Evonik Industries) were an anatase/rutile (8:2) mixture with an average size of 21 nm. Hydrogenases were handled anaerobically in a VAC glovebox (O_2 less than 2 ppm). The [NiFeSe]-hydrogenase from *Desulfomicrobium baculatum* (DSM 1743) was purified using a previously published method.[28] The purity of the enzyme preparation was assessed by mass spectrometry, with homogeneous peaks at 55 086 and 30 842 Da for the large and small subunit, respectively. The isoelectric point of *Db* [NiFeSe]-H (Ip = 5.4 ± 0.2) was determined by performing isoelectric focusing using a Phast System apparatus (GE Healthcare). Samples of other hydrogenases were kindly provided by Prof. S. Albracht, Prof. W. Lubitz, Prof. T. Happe, Dr. A. Parkin, M. Lukey, and S. Stripp.

Electrochemical Measurements. Thin film TiO_2 nanoparticle working electrodes (0.25 cm^2 TiO_2 surface area) on either indium-doped tin oxide (ITO) or fluorine-doped tin oxide (FTO)-coated

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glass slides were prepared by the doctor's blade technique as described in the literature.[29] The hydrogenase films were prepared by spreading 4 μL of enzyme solution across the TiO_2 surface, whereupon the electrodes were immersed into the electrolyte solution after approximately 1 min. Protein film voltammetry (PFV) was performed using an Autolab PGSTAT 20 electrochemical analyzer equipped with a digital staircase scan generator and controlled by GPES software (Eco Chemie). A two-compartment three-electrode glass electrochemical cell was used with a Pt auxiliary electrode and a saturated calomel reference electrode (SCE) held in a side arm separated from the main cell compartment and linked by a Luggin capillary. The cell was thermostatted at 25 °C by a water jacket connected to a water flow thermostat. Potentials were corrected to correspond to the standard hydrogen electrode (SHE) scale by adding +0.24 V to the experimental values.[30] Cell solutions were saturated with either H_2 (Premier grade, Air Products) or Ar (Oxygen Free, BOC). Control experiments established the following: blank cyclic voltammograms with the TiO_2 working electrodes without adsorbed enzyme did not show significant amounts of Faradaic currents due to H^+ reduction, adsorption of *Db* [NiFeSe]-H on FTO electrodes (no TiO_2) showed only small currents, and direct adsorption of *Db* [NiFeSe]-H on ITO slides showed only a negligible enzyme-electrocatalytic response.

Adsorption of Photosensitizers and Hydrogenases to TiO_2. Each dye (0.20 μmol) was dissolved in either 0.4 mL of water for complexes **1**−**3**, or TEOA buffer (25 mM) at pH 7 for complexes **4** and **5**. Ten milligrams of TiO_2 was sonicated in 3.6 mL of the respective solvent, and the dye solution was then added to the stirred dispersion. The mixture was left stirring overnight under protection from light, centrifuged, and the filtered clear supernatant was then analyzed by UV−vis spectrophotometry (Perkin-Elmer Lambda 750S spectrophotometer). The amount of adsorbed dye was quantified by the absorbance difference at λ_{max} between the initial and the filtered supernatant dye solution. Desorption of the photosensitizers **1**−**3** was studied by redispersing the centrifuged dye-sensitized TiO_2 particles in fresh TEOA buffer (4 mL, pH 7, 25 mM) and stirring the resulting dispersion for 24 h. The amount of desorbed complex was quantified by spectrophotometry on the centrifuged and filtered supernatant. Attachment of hydrogenases to the TiO_2 nanoparticles was quantified by analyzing the absorbance difference at 280 nm of enzyme solutions (2 mL, 3 μM in pH 7 TEOA buffer) before and after stirring for 15 min with TiO_2 (300 mg). The extinction coefficients for *Db* [NiFeSe]-H are 53 mM^{-1} cm^{-1} at 400 nm and 190 mM^{-1} cm^{-1} at 280 nm.

Assembly of Photocatalytic H_2 Production Particles. The dye-sensitized TiO_2 particles were prepared by the following standard procedure, unless otherwise noted. A mixture of dispersed TiO_2 nanoparticles (50 mg) and photosensitizer (1.0 μmol) was stirred in 5 mL of water for **1**−**3** or TEOA buffer (25 mM, pH 7) for **4** and **5** overnight. The particles were separated from the supernatant solution by centrifugation and dried. A 5 mg aliquot of these particles was then sonicated for 5 min in TEOA (4.5 mL) buffer in a Pyrex pressure reaction vessel (total volume 9 mL) to disaggregate the particles. The hydrogenase (20 μL of 1 μM solution) was added to the dispersion in an anaerobic glovebox, and the mixture was stirred for 15 min to enable enzyme adsorption to occur. The reaction vessel was sealed tightly with a rubber septum and removed from the glovebox for the light experiments.

Photocatalytic H_2 Production. Photocatalytic experiments were performed with either a slide projector light source (Kodak Carousel S-AV 1010) featuring a 250 W (24 V) tungsten halogen lamp (Philips, light intensity 45 mW cm^{-2} measured using a Melles Griot Broadband Power/Energy Meter 13PEM001) or real outdoor sunlight in Oxford, UK. The UV radiation of the halogen lamp was filtered out with a 420 nm cutoff filter (UQG Optics). The gently stirred and light-protected reaction vessel was flushed for 15 min with 2% CH_4 in N_2. Methane acts as an internal standard for H_2 quantification (see below). The reaction vessel was thermostatted with a water circulator connected to a water-jacket reservoir. The amount of photogenerated H_2 was detected and quantified by headspace gas analysis with an Agilent 7890A Series gas chromatograph (GC) equipped with a 5 Å molecular sieve column (N_2 carrier gas at a flow rate of 3.5 mL min^{-1}) held isothermally at 40 °C, and a thermal conductivity detector. The instrument was calibrated with various known amounts of H_2 in a mixture of CH_4 (2%) in N_2, and the linearity and stability of the instrument and method were checked regularly. The total irradiation time for each light experiment was 4 h, with 10 μL samples of the headspace gas removed twice for GC analysis after 0.5, 1, 1.5, 2, 3, and 4 h. Each experiment was carried out three times, and the standard deviation was calculated using an unweighted formula.[31] In the absence of either light, chromophore, semiconductor, or enzyme, only small traces or no H_2 were detected under the same experimental conditions.

Air-Tolerance of Photocatalytic Particles. The stability toward air of *Db* [NiFeSe]-H attached on TiO_2 nanoparticles was studied as follows. The enzyme (20 μL of a 1 μM solution) was added to a sonicated solution of TiO_2 (5 mg) in TEOA buffer (pH 7, 4.4 mL) in an anaerobic glovebox, whereupon the dispersion was stirred for 15 min. The particles were then stirred gently during exposure to air for varying amounts of time at 23−25 °C. The enzyme−TiO_2 particle dispersion was sealed with a rubber septum, then purged for 15 min with 2% CH_4 in N_2, whereupon RuP (0.1 μmol in 0.1 mL of buffer) was added. The vessel was purged for a further 15 min, and the enzyme activity was assayed by photocatalysis in visible light ($\lambda > 420$ nm) illumination. The total irradiation time for each of these light experiments was 1 h, and gas samples were quantified by gas chromatography after 0, 15, 30, 45, and 60 min. The H_2 production rate was calculated from the data measured after 30 and 60 min irradiation. Stability in air of free *Db* [NiFeSe]-H in TEOA was studied by a similar procedure, with the following modifications: free *Db* [NiFeSe]-H was gently stirred in TEOA buffer (4.0 mL) in air, whereupon a dispersion of TiO_2 (5 mg in 0.4 mL of buffer) was added after exposing the enzyme to air. The resulting dispersion was purged for 15 min during stirring, and RuP (0.1 μmol in 0.1 mL of buffer) was added. The assembled system was purged for an additional 15 min period, followed by visible light irradiation.

Results and Discussion

The principle of the prototype system depicted in Figure 1 is that visible light excites the photosensitizer, which injects one electron at a time into the TiO_2 conduction band (the oxidized dye is recovered by the sacrificial donor); the electrons are then transferred into the enzyme, via a series of FeS-clusters linking the enzyme's surface to the buried active site at which protons from the aqueous solution are reduced to H_2.[22] The light-driven system mediates the cathodic half-reaction of water splitting.

Stable adsorption of a hydrogenase in an electroactive form on the TiO_2 surface is a prerequisite for photocatalytic H_2 production in this kind of experiment. Previous voltammetry experiments showed that *Db* [NiFeSe]-H adsorbed on a TiO_2 electrode gives a high current density combined with a slow desorption/inactivation rate: only about 20% of activity was lost after 2 days when storing the electrode in a pH 7 buffer solution at room temperature under anaerobic conditions.[22]

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(31) Treatment of data: Considering a sample of n observations x_i, the unweighted mean value (x_u) with its standard deviation (σ) was calculated using the following equations: $x_u = \Sigma_i x_i/n$, $\sigma = \{\Sigma_i(x_i - x_u)^2/[n(n-1)]\}^{1/2}$.

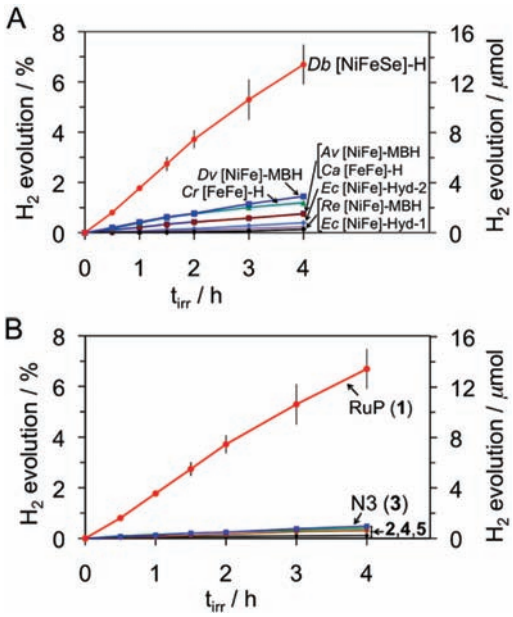

Figure 2. The effect of different hydrogenases (A) and photosensitizers (B) on visible light-driven H_2 evolution (in percent and μmol of H_2 accumulated in the reaction vessel headspace) during 4 h irradiation in TEOA buffer (25 mM, 4.5 mL) at pH 7 and 25 °C. (A) Various hydrogenases (20 μL of 1 μM enzyme solutions have been used) attached on RuP-sensitized TiO_2 (0.1 μmol on 5 mg). (B) Various photosensitizers (0.1 μmol) co-attached with *Db* [NiFeSe]-H (20 μL of 1 μM) to TiO_2 (5 mg). See Table 2 for a description of compounds **1**−**5**.

Nanoparticles modified with different hydrogenases and photosensitizers were assembled by a routine that we will refer to as our standard procedure (unless otherwise stated), and the results are summarized in Figure 2. The standard procedure consisted of the following steps: a 20 μL aliquot of 1 μM hydrogenase solution was added to a dispersion of TiO_2 that had been derivatized with the photosensitizer (0.1 μmol of dye on 5 mg of metal oxide) in 4.5 mL of 25 mM TEOA[32] buffer, pH 7. The tube was purged with N_2 containing 2% CH_4 as a calibrant for quantitative GC analysis. Production of H_2 at 25 °C, using TEOA as the sacrificial electron donor, was then driven by visible light irradiation ($\lambda > 420$ nm) from the focused 250 W tungsten−halogen lamp with the projector lens positioned 3 cm from the reaction vessel. As a benchmark, particles consisting of *Db* [NiFeSe]-H attached on RuP-sensitized TiO_2 assembled by this standard procedure show an initial turnover frequency of 50 (mol H_2) s^{-1} (mol total hydrogenase)$^{-1}$, based on the average H_2 production within the first hour of irradiation, and an absolute H_2 production rate of 3.56 μmol h^{-1} (Table 1). This rate corresponds to an accumulation of 6.7% H_2 in the headspace (total volume of headspace: 4.5 mL) of the reaction vessel after 4 h. We first evaluated different hydrogenases on TiO_2 sensitized with complex **1** (RuP) (Figure 2A), and then we evaluated different photosensitizers on TiO_2 to which *Db* [NiFeSe]-H is coadsorbed (Figure 2B).

The Effectiveness of Various Hydrogenases in Photocatalytic H_2 Production. With the exception of *Cr* [FeFe]-H (see below), approximately linear H_2 evolution rates were obtained, over a period of 4 h, for hydrogenases attached on RuP-sensitized TiO_2. However, their activities varied significantly (Figure 2A and Table 1). The best result, by a considerable margin, was obtained with *Db* [NiFeSe]-H. The O_2-tolerant hydrogenases *Ec* [NiFe]-Hyd-1[33] and *Re* [NiFe]-MBH,[34] which are known to be poor H_2 producers even under a non-H_2 atmosphere, did not substantially enhance H_2 evolution when compared to a control experiment without hydrogenase. Better results were obtained with *Ca* [FeFe]-H, *Cr*[FeFe]-H, *Dv* [NiFe]-MBH, and *Av* [NiFe]-MBH even though these enzymes (particularly the [FeFe]-hydrogenases)[35] are O_2-sensitive. The [FeFe]-hydrogenases showed poor reproducibility ($\sigma > 30\%$) or less stability (approximately 20% activity loss h^{-1}) in the photocatalytic experiments. This was most likely due either to irreversible O_2-induced damage or to photodamage.[36] A hydrogenase from *E. coli*, *Ec* [NiFe]-Hyd-2, which exhibits (relative to other [NiFe]-hydrogenases) a high O_2-tolerance, low product (H_2) inhibition, and excellent electrocatalytic H_2 production activity on PGE electrodes,[37] was also tested. This hydrogenase was recently demonstrated to be an excellent cocatalyst for the water-gas-shift reaction on graphite platelets[21] and was expected to be a successful catalyst. However, *Ec* [NiFe]-Hyd-2 showed only marginal H_2 evolution rates as compared to the benchmark enzyme, *Db* [NiFeSe]-H.

Such a large difference in H_2 production activity between *Ec* [NiFe]-Hyd-2 and *Db* [NiFeSe]-H when attached to RuP-sensitized TiO_2 was not anticipated because both enzymes are good H_2 producers, even in the presence of some O_2. A necessary condition for a hydrogenase to operate effectively in this system is its interaction with TiO_2 in a stable, electrocatalytically active manner, that is, in the correct configuration allowing for direct electron transfer at the metal−oxide/enzyme interface. This was not the case for *Ec* [NiFe]-Hyd-2 because even when using more than a 100-fold excess (>100 μM) of enzyme the activity increased only marginally to 0.39 μmol H_2 h^{-1}. All of our attempts to adsorb *Ec* [NiFe]-Hyd-2 productively on TiO_2 electrodes failed as only negligible Faradaic currents were obtained in voltammetric experiments with this enzyme. On the other hand, *Db* [NiFeSe]-H consistently exhibited stable attachment to TiO_2 in a highly electrocatalytic state, a property we term 'titaniaphilicity'. We carried out experiments to compare the quantities of enzymes adsorbed from solution, using the optical density at 280 nm. Stirring *Db* [NiFeSe]-H and *Ec* [NiFe]-Hyd-2 (2 mL of 3 μM solution) in TEOA buffer at pH 7 for 15 min in the presence of 300 mg TiO_2 results in the adsorption of approximately 90% and 75% enzyme, respectively. Consequently, a higher quantitative adsorption cannot be solely responsible for the higher activity of *Db* [NiFeSe]-H, and its special suitability must be explained by a qualitative measure, that is, adsorption to TiO_2 in a stable, electroactive orientation.

The Effectiveness of Various Photosensitizers in Photocatalytic H_2 Production. The primary requirements of a suitable photosensitizer are efficient visible light harvesting and ultrafast electron injection, in this case from an excited state into the

(32) TEOA buffer allows for considerably increased long-term stability (almost constant H_2 evolution rate) as compared to EDTA (see ref. 22). No decoloration (bleaching) of the photo-sensitizer (RuP) attached to TiO_2 is observed even after 8 h irradiation (see the Supporting Information).

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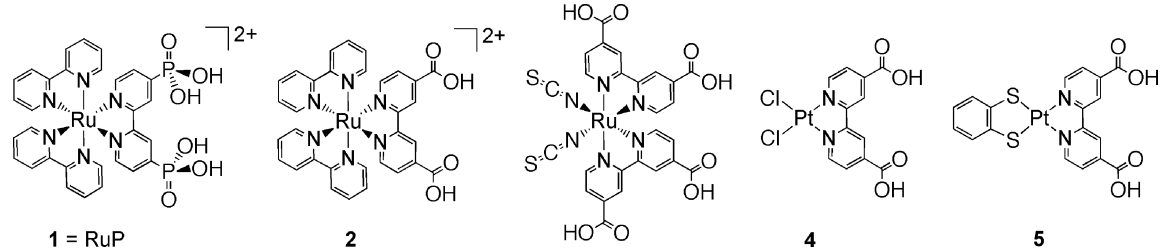
Table 1. Photocatalytic H_2 Production with Various Hydrogenases Attached to RuP-Sensitized TiO_2 Nanoparticles (0.1 μmol of Dye on 5 mg of Nanoparticles) in TEOA Buffer at pH 7.0 and 25 °C[a]

hydrogenase	$H_2 \pm \sigma$[b]/μmol h^{-1}	H_2 (4 h) $\pm \sigma$[c]/%
Good H_2 Producer and Some O_2-Tolerance		
Desulfomicrobium baculatum (*Db*) [NiFeSe]-H	3.56 ± 0.17	6.69 ± 0.56
Escherichia coli (*Ec*) Hyd-2	0.14 ± 0.02	0.39 ± 0.03
Ec Hyd-2 (concentrated)	0.39 ± 0.04	0.69 ± 0.04
Good H_2 Producer but Highly O_2-Sensitive		
Desulfovibrio vulgaris (*Dv*) [NiFe]-MBH Miyazaki	0.85 ± 0.07	1.44 ± 0.12
Dv [NiFe]-MBH (concentrated)	2.5 ± 0.2	4.2 ± 0.4
Allochromatium vinosum Miyazaki F (*Av*) [NiFe]-MBH	0.44 ± 0.01	0.74 ± 0.04
Clostridium acetobutylicum (*Ca*) [FeFe]-H	0.43 ± 0.18	0.76 ± 0.39
Chlamydomonas reinhartii (*Cr*) [FeFe]-H	0.84 ± 0.06	1.20 ± 0.04
Some O_2-Tolerance but Poor H_2 Producer		
Ralstonia eutropha H16 (*Re*) [NiFe]-MBH	0.11 ± 0.01	0.24 ± 0.01
Escherichia coli (*Ec*) Hyd-1	0.06 ± 0.02	0.14 ± 0.01

[a] 20 μL of 1 μM solution was used for *Db* [NiFeSe]-H (standard condition), whereas for the other enzymes 20 μL aliquots of a 1−10 μM solution were employed. Concentrated samples were 20 μL of 100 μM solution. [b] Mean amount of H_2 produced within the first hour of irradiation ($\pm\sigma$, standard deviation). [c] H_2 accumulated in the headspace of the reaction vessel after 4 h irradiation.

Table 2. Properties of Ruthenium and Platinum Photosensitizers and Their Efficiency for Visible Light-Driven H_2 Production



compound	λ_{max}, nm[a] (ε/mM^{-1} cm^{-1})	adsorption on TiO_2,[b] %	$H_2 \pm \sigma$,[c] μmol h^{-1}	H_2 (4 h) $\pm \sigma$,[c] %
1 (RuP)	457 (8.5)	>98	3.56 ± 0.17	6.69 ± 0.56
2	445 (10.0)	<2	0.13 ± 0.05	0.31 ± 0.08
3 (N3)	500 (9.3)	<2	0.24 ± 0.06	0.48 ± 0.04
4	372 (3.7)	>90	0.30 ± 0.10	0.40 ± 0.10
5	506 (5.1)	~50	0.11 ± 0.03	0.12 ± 0.04

[a] UV−vis spectra recorded in TEOA buffer (25 mM) at pH 7 and room temperature. [b] Total amount of Ru or Pt complex attached on TiO_2 after adsorption from an aqueous solution overnight and stirring the dye-sensitized TiO_2 particles in fresh TEOA buffer (25 mM) at pH 7 and room temperature for 24 h. [c] Standard conditions with the corresponding dye being employed for visible light-driven H_2 production experiments: *Db* [NiFeSe]-H (20 μL of 1 μM solution) and photosensitizer (0.1 μmol) co-attached to TiO_2 (5 mg) in TEOA (25 mM, 4.5 mL) buffer at pH 7 and 25 °C, followed by irradiation with a tungsten halogen lamp (250 W, $\lambda > 420$ nm) under anaerobic conditions ($\pm\sigma$, standard deviation).

conduction band of TiO_2.[38] In the absence of dye, the large band gap of TiO_2 (3.2 eV) precludes visible light absorption, and photocatalysis is only feasible with UV-irradiation. Although UV-light promotes sacrificial H_2 production with hydrogenases on TiO_2,[39,40] its inefficient solar light collection (UV light constitutes only a small fraction of sunlight) and formation of reactive (highly oxidizing) electron holes in the valence band of TiO_2 are major limitations in the absence of dye. Electron holes in TiO_2 cause photodegradation of adsorbed amino acids[41] and therefore preclude long-term stability of attached enzymes.

Platinum photosensitizers have emerged as potential alternatives to ruthenium dyes,[42,43] and a series of Ru and Pt bipyridyl complexes were tested for their suitability for visible light ($\lambda >$ 420 nm)-driven H_2 production when attached to TiO_2 nanoparticles modified with *Db* [NiFeSe]-H under standard conditions (Figure 2B, Table 2). Complex **1** (RuP) clearly emerged as the only suitable photosensitizer among this group. This can be rationalized on the basis that a suitable photosensitizer attached to TiO_2 must fulfill several requirements, including (i) an absorption band in the visible spectrum, (ii) stable attachment to TiO_2 under the experimental conditions employed, (iii) efficient charge separation, and (iv) long-term stability upon irradiation. The absorbance maxima for the orange-yellow Ru complexes **1** and **2**, dark brown Ru complex **3**, and dark blue Pt compound **5** lie in the visible spectral range, whereas the pale yellow Pt complex **4** only absorbs minimally with a shoulder in the visible region and is therefore less suitable.

The anchoring group of the photosensitizer governs its stability on TiO_2. Photosensitizers **1**−**5** were reacted with TiO_2 nanoparticles in slightly acidic or neutral aqueous solutions (0.1 μmol of dye per 5 mg of TiO_2) overnight; the dye-derivatized

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particles were separated by centrifugation, then redispersed and stirred for 24 h in fresh 25 mM TEOA buffer at pH 7 and room temperature. Electronic absorption spectroscopy revealed that RuP (**1**), which contains a phosphonate linker, binds strongly to TiO_2 even in buffered pH 7 solution, as indicated by decoloration of the supernatant solution with concomitant permanent color transfer to TiO_2. The Ru complexes **2** and **3** (N3) with carboxylate anchoring groups are not suitable as photosensitizers because they desorb rapidly from TiO_2 at neutral pH.[44] About 50% of complex **5** is bound to TiO_2 in TEOA buffer at pH 7 (see the Supporting Information).

Thus, under the experimental conditions employed, only complexes **1** and **5** are potentially useful photosensitizers because they show good-to-excellent attachment on TiO_2 and light absorption in the visible region. Although RuP-sensitized TiO_2 electrodes in TEOA buffer at pH 7.0 showed high photocurrent densities,[22] TiO_2 electrodes derivatized with complex **5** showed only a minor response upon visible light irradiation, and complex **5** was therefore also deemed unsuitable.

Special Suitability of *Db* [NiFeSe]-H as the H_2 Production Catalyst on TiO_2. [NiFeSe]-hydrogenases are a subclass of [NiFe]-hydrogenases in which one of the terminal cysteine ligands to nickel is replaced by selenocysteine.[45] As compared to standard [NiFe]-hydrogenases, *Db* [NiFeSe]-H exhibits the following properties: (i) good H_2 production activity (catalytically more biased toward H_2 production than other [NiFe]-hydrogenases); (ii) rapid reactivation at low potentials after O_2-induced inactivation; and (iii) the ability to operate in the presence of $\leq 1\%$ O_2.[46] Each of these characteristics is important for practical, benchtop photo H_2 production, particularly in water splitting itself where O_2 is also formed. However, good H_2 production ability and O_2 tolerance alone are clearly not sufficient for optimal H_2 production, as illustrated by the poor performance of *Ec* [NiFe]-Hyd-2. Electrochemical experiments showed that this enzyme does not interact productively with TiO_2. Aside from specific properties due to replacement of a cysteine by a selenocysteine at the active site, the overall structural and electrical properties of the hydrogenase are also clearly important. In most of our experiments, the photocatalytic particles were assembled and used at neutral pH. The isoelectric points of *Db* [NiFeSe]-H and TiO_2 (P-25) nanoparticles are 5.4 and 6.0, respectively. A simple vacuum electrostatic model calculated with PyMOL[47] reveals a negatively charged surface patch around the distal iron−sulfur cluster. This indicates that the interaction between *Db* [NiFeSe]-H and the particle is likely to be controlled by localized polar interactions between side-chain carboxylates and a number of Ti−O(H) sites rather than by overall electrostatic interactions.[48] No substantial improvement of photo H_2 production activity was obtained when adding the cyclic cationic polypeptide polymyxin to the system. Strong interactions between TiO_2 and aspartate or glutamate carboxylates have been reported,[49] and free glutamate binds to TiO_2 even in alkaline solution (pH 8) despite the expected electrostatic repulsion.[50] Experiments carried out with *Ec* [NiFe]-Hyd-2 (Ip = 4.5)[51] at pH 6 showed that no marked improvement was obtained by using a lower pH in an attempt to compensate for the lower Ip value.

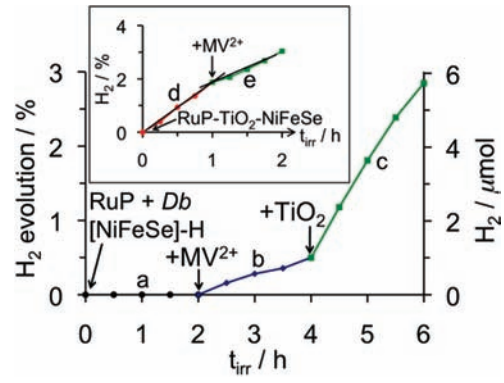

Figure 3. Direct electron transfer is faster than diffusion-controlled electron transfer. Visible light-driven H_2 production with *Db* [NiFeSe]-H (20 μL of 1 μM solution) and RuP (0.1 μmol) in TEOA buffer (25 mM) at pH 7.0 and 25 °C (a, black trace), after addition of 1 μmol of methyl viologen (b, blue trace), and after further addition of 5 mg of TiO_2 (c, green trace). Inset: Photocatalytic H_2 production under standard conditions (d, red), in the additional presence of MV^{2+} (1 μmol, e, green).

Direct versus Diffusion-Controlled Electron Transfer. Diffusion-controlled sacrificial photo H_2 production involving electron transfer between a hydrogenase and a photosensitizer mediated by a soluble redox mediator (e.g., methyl viologen, MV^{2+}) is well established.[52] Furthermore, MV^{2+} enhances the H_2 production rate under UV-band gap irradiation by a factor of 2−100 when added to a TiO_2−hydrogenase suspension.[39,40]

Evidence for particularly proficient direct electron transfer between TiO_2 and the attached *Db* [NiFeSe]-H is provided in Figure 3. No detectable amounts of H_2 were formed when irradiating a TEOA solution (4.5 mL, 25 mM, pH 7) of RuP and *Db* [NiFeSe]-H with visible light at 25 °C (trace a). Addition of MV^{2+} (0.26 mg, 1 μmol) resulted in an activity of approximately 0.44 μmol H_2 h^{-1} (trace b), and further addition of TiO_2 (5 mg) increased H_2 evolution activity 6-fold (2.48 μmol H_2 h^{-1}; trace c). Therefore, electron transfer between RuP and *Db* [NiFeSe]-H via TiO_2 is faster than that via the bimolecular MV^{2+} electron relay. This observation suggests that attachment of the enzyme to the TiO_2 surface is highly competent in terms of electronic coupling, noting that only 20 pmol of enzyme is used. Addition of (1 μmol) MV^{2+} to *Db* [NiFeSe]-H attached on RuP-sensitized TiO_2 under standard conditions resulted in a noticeable decrease in the H_2 evolution activity of the system (Figure 3, inset). It appears that some electrons are removed from the integrated system by reaction with methyl viologen (as indicated by the dark blue color of the solution). The decreased availability of electons for proton reduction by the hydrogenase or even the absorption of incident visible-light photons by the deeply colored, reduced MV^{2+} are presumably responsible for the decreased activity.

Aerial Stability of *Db* [NiFeSe]-H on TiO_2. After establishing the superior electrocatalytic activity of *Db* [NiFeSe]-H attached to TiO_2, we investigated its robustness under more extreme

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conditions, in the presence of air. The hybrid system was assembled under standard conditions, but was exposed to air by gentle stirring prior to illumination under 2% CH_4 98% N_2. Initially, the photocatalytic particles were stirred in air while being exposed to daylight. These experiments showed highly irreproducible results, and about 50% of activity was lost after 0.5 h exposure to air. Protection of the dispersion from light by an Al foil resulted in a greatly increased stability of the particles toward air, and 50% of activity was lost only after approximately 15 h.

Irradiation (250 W, $\lambda > 420$ nm) of the particles in air with a tungsten halogen lamp resulted in their instantaneous and complete inactivation within 2 min (see the Supporting Information) indicative of O_2-dependent photodecomposition. Addition of 1 equiv of RuP (0.09 mg, 0.1 μmol) to this inactive dispersion did not reactivate it, but addition of 1 equiv of *Db* [NiFeSe]-H (20 μL of 1 μM solution) almost quantitatively restored the H_2 production rate (2.85 μmol h^{-1}). Irradiation of *Db* [NiFeSe]-H attached on TiO_2 for 30 min in air followed by addition of RuP under anaerobic conditions resulted in good H_2 formation rates (3.05 μmol h^{-1}), thereby excluding light sensitivity of the enzyme on TiO_2 as the main reason for inactivation. Thus, the air-exposed enzyme is completely degraded within minutes under visible light illumination only in the presence of the photosensitizer RuP. Reactive oxygen species formed upon reduction of O_2 by conduction band electrons in TiO_2 are very likely responsible for enzyme damage.[53] In this case, RuP is sufficiently active that conduction band electrons are generated even under laboratory daylight.

Because of the extreme light sensitivity of the fully assembled particle system in air, we tested the robustness of *Db* [NiFeSe]-H (20 μL of 1 μM solution) either free in solution or attached to TiO_2 (5 mg) under air in the absence of RuP and light. The residual activity of *Db* [NiFeSe]-H was quantified by a photocatalytic enzyme activity assay using the following procedure: free *Db* [NiFeSe]-H and a dispersion of *Db* [NiFeSe]-H attached to TiO_2 were exposed to air for a certain amount of time in two separate vials. Next, to the first vial, 0.1 μmol of RuP and TiO_2 were added to the enzyme solution, whereas to the second vial, RuP was added to the already-established *Db* [NiFeSe]-H TiO_2 dispersion, both additions being made under anaerobic conditions. The H_2 evolution rates were measured after visible light irradiation.

Plots of remaining enzyme activity versus air exposure time for free *Db* [NiFeSe]-H and *Db* [NiFeSe]-H on TiO_2 are shown in Figure 4A. The free hydrogenase is slightly more active in this assay (50% activity is lost, $\tau_{1/2}$, after 43 h) than the hydrogenase attached on TiO_2 ($\tau_{1/2} = 27$ h). The difference in activity after exposure to air in these two assays arises at least partially from loss of the electroactive enzyme from the TiO_2 surface.

Protein film voltammetry was used as a complementary method (an electrochemical assay) to test the air sensitivity of the enzyme after exposing a TiO_2 electrode covered with a *Db* [NiFeSe]-H film in buffered solution to air. Figure 4B shows the voltammetric response under Ar and H_2 atmospheres at 25 °C before and after exposing the electrodes to air. The observed current in each case is proportional to the electrocatalytic activity of the enzyme, and after exposure to air for 2 and 12 h, approximately 10% and 50% of activity are lost, respectively. Both assays work because they impose a negative (reducing)

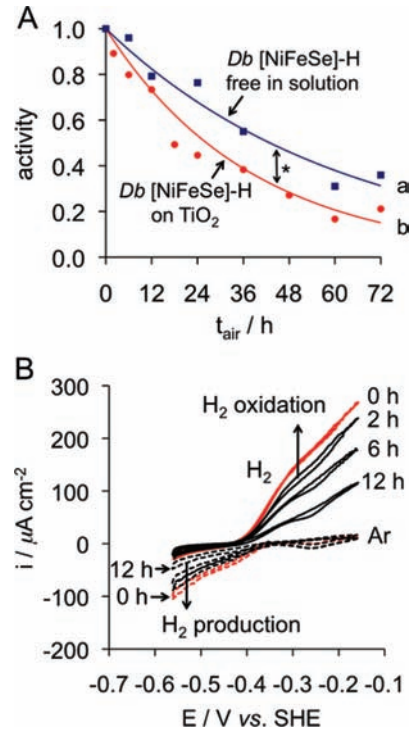

Figure 4. (A) Plot of hydrogenase activity against air exposure time when exposing *Db* [NiFeSe]-H (20 μL of 1 μM solution) free in solution (a) and attached on TiO_2 (b) to air at 25 °C and TEOA buffer at pH 7.0. The activity was measured after exposure to air by an anaerobic photocatalytic H_2 production assay under standard conditions upon addition of TiO_2 (a) and RuP (a and b). The solid lines represent exponential fits of experimental data: activity $= e^{-0.016 \times t_{air}(h)}$, $R = 0.97$ (for a); activity $= e^{-0.026 \times t_{air}(h)}$, $R = 0.96$ (for b). The difference in activity, as indicated by the asterisks, is due to film loss from the enzyme-modified particles in (b) during exposure to air. (B) Protein film voltammograms of *Db* [NiFeSe]-H adsorbed on a stationary TiO_2 electrode in TEOA (25 mM) and NaCl (100 mM) at pH 7 and 25 °C under a H_2 (solid lines) or Ar (dashed lines) atmosphere before (red traces) and after (black traces) exposing the electrodes to air for 2, 6, and 12 h.

potential on the hydrogenase, which enables the enzyme to be rapidly reactivated after aerobic inactivation.[46]

Integrated Studies: The Rate-Limiting Component. *Db* [NiFeSe]-H and RuP were the best combination from among a series of hydrogenases and photosensitizers considered for sacrificial solar H_2 production on TiO_2 nanoparticles. The effects of varying amounts of RuP, TiO_2, and hydrogenase under otherwise standard conditions were studied to identify the activity-limiting component (Figure 5, Table 3).

We observed that if a high concentration of RuP is used, the order in which RuP and enzyme are introduced to the TiO_2 dispersion becomes very important. Sensitizing the TiO_2 particles with excess RuP (0.5 μmol per 5 mg of TiO_2) resulted in the adsorption of only 48% of the total RuP as measured by electronic absorption spectrophotometry (see the Supporting Information). This effect is attributed to saturation coverage at a density consistent with one monolayer (0.6 $\pm$ 0.2 RuP molecules per nm^2 TiO_2). Subsequent addition of *Db* [NiFeSe]-H to this RuP-saturated TiO_2 did not produce an active system (Figure 5A, trace d). On the other hand, adsorbing *Db* [NiFeSe]-H on TiO_2 prior to addition of 0.5 μmol of RuP resulted in photocatalytic particles with full H_2 production activity (trace a). The remaining 52% of soluble RuP neither enhanced H_2 production nor displaced the enzyme from the semiconductor

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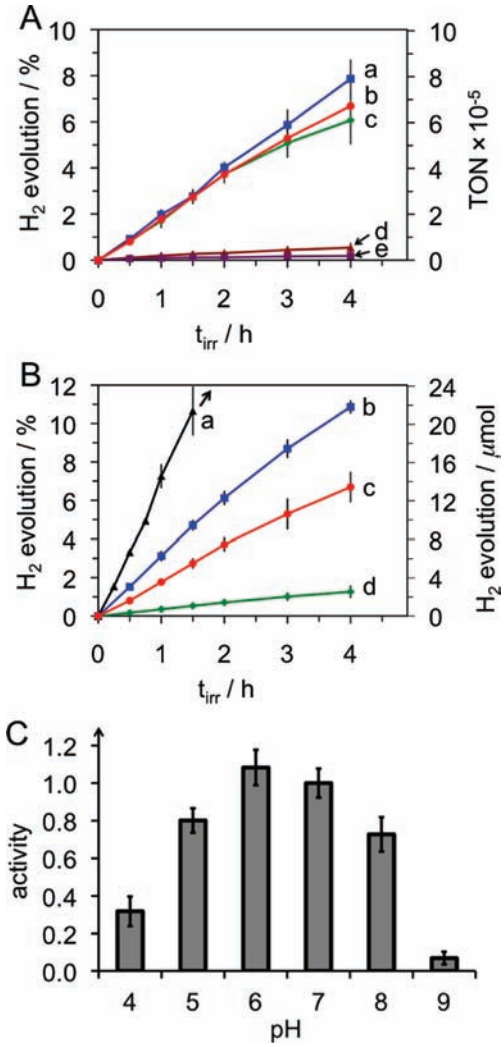

Figure 5. Visible light-driven H_2 production with varying amounts of RuP (A) and *Db* [NiFeSe]-H (B) co-attached to TiO_2 (5 mg) upon visible light irradiation in TEOA buffer (25 mM, 4.5 mL) at pH 7 and 25 °C. The effect of varying pH is shown in (C). Standard deviations are given as vertical black lines. (A) Amounts of RuP; (a) 0.5 μmol (RuP added after hydrogenase); (b) 0.1 μmol; (c) 0.02 μmol; (d) 0.5 μmol (RuP added before hydrogenase); (e) 0.1 μmol in phosphate/TEOA (both 25 mM) buffer. Twenty microliters of 1 μM *Db* [NiFeSe]-H was used (a−e). In (d) and (e), the TiO_2 surface is saturated with RuP or phosphate, respectively, preventing attachment of *Db* [NiFeSe]-H and thus the assembly of an operational system. (B) Concentrations of *Db* [NiFeSe]-H (20 μL); (a) 100 μM; (b) 5 μM; (c) 1 μM; (d) 0.2 μM; 0.1 μmol RuP have been used in (a)−(d). (C) pH-dependent photocatalytic H_2 evolution activity with RuP (0.1 μmol) and *Db* [NiFeSe]-H (20 μL, 1 μM) on TiO_2 (5 mg) in TEOA buffer (25 mM) at 25 °C relative to its activity at pH 7 (standard condition).

surface (at least within 4 h) as indicated by the fact that the H_2 production rate was unchanged from that observed using a lower amount of RuP. A plausible explanation for this behavior is that RuP, at high levels, forms a dense coverage on the oxide, leaving no space for the much larger enzyme molecules to adsorb. On the other hand, even if *Db* [NiFeSe]-H were adsorbed to saturation, there should always remain sites at which the much smaller RuP complex could bind. This explanation is supported by the very different results obtained using lower concentrations of RuP. Decreasing the amount of RuP to 0.02 μmol on 5 mg of TiO_2 resulted in no marked effect on the photocatalytic H_2 evolution rate, indicating that electron injection from RuP into TiO_2 is very efficient and that neither the amount of RuP nor the density of conduction band electrons are determining factors for H^+-reduction under most conditions (traces b and c). At these lower loadings, the importance of introducing the photosensitizer after attaching the enzyme was much less marked.

Figure 5A (trace e) also shows the effect of phosphate binding to the TiO_2 surface. It is well-known that phosphate has a high affinity for TiO_2.[54] Adsorbing *Db* [NiFeSe]-H on RuP-sensitized TiO_2 under standard conditions in the presence of a pH 7 phosphate/TEOA 25 mM buffer resulted in only marginally active particles. Phosphate therefore acts as a strong inhibitor by blocking the binding sites on TiO_2 for *Db* [NiFeSe]-H as well as by displacing RuP, as indicated by formation of a yellow supernatant solution.

In contrast to using the photosensitizer at subsaturation amounts, variation of the amount of TiO_2 and the concentration of the hydrogenase had considerable effects on H_2 production efficiency. The photocatalytic activity was enhanced 2.5-fold when the amount of RuP-sensitized TiO_2 was increased from 1 to 5 mg. On the other hand, an additional increase to 25 mg resulted in only a 20% enhancement in photocatalytic activity (see the Supporting Information). With low amounts of RuP-sensitized TiO_2, little light is absorbed, and more enzyme molecules compete for each TiO_2 aggregate and its conduction band electrons, whereas with more RuP-sensitized TiO_2 the incident photons are fully absorbed by the dense dispersions, and fewer enzyme molecules are attached per TiO_2 nanoparticle, resulting in increased H_2 evolution.

Figure 5B shows how the activity of the photocatalytic particles varies with enzyme concentration. When using very dilute enzyme samples (20 μL of 0.2−1 μM solution) of [NiFeSe]-hydrogenase, a 5-fold increase in enzyme concentration results in a 5-fold enhancement in H_2 production. Thus, a constant turnover frequency of approximately 50 s^{-1} (per mol total hydrogenase) is obtained with low amounts of enzyme. The increase in H_2 production rate is less pronounced when a larger amount of enzyme (20 μL) is added: increasing the enzyme concentration from 1 to 5 μM and from 5 to 100 μM results in a 1.7 and 2 fold-enhancement of photocatalytic H_2 production activity with a corresponding decrease in turnover frequency to approximately 17 and 2 s^{-1}, respectively. This decrease in enzymatic H_2 production rate indicates that other factors start to compete with the rate-limiting proton reduction at a more concentrated enzyme concentration and increased enzyme to TiO_2 ratio.

Integrated Studies: Stability and Performance. The effects on photocatalytic H_2 production of varying the pH and temperature were investigated to assess the practical limitations and applicability of the system (Table 3). The system performed well over a wide pH range, reaching a maximal turnover frequency of 54 ± 6 s^{-1} at pH 6.0 (Figure 5C), which is close to the Ip values of TiO_2 and *Db* [NiFeSe]-H (see above). At either pH 5 or 8, significant H_2 production rates were still observed (37 ± 1 and 36 ± 5 s^{-1}, respectively). Further deviation from neutrality resulted in considerable inactivation of the system with 18 ± 3 and 4 ± 2 s^{-1} rates, at pH 4 and 9, respectively. The almost complete lack of activity at pH 9 is consistent with the lack of H_2 production activity of the enzyme under such proton-poor conditions and correlates also with RuP desorption from TiO_2 as indicated by coloration of the supernatant solution and discoloration of the particles. The pH value increased only slightly during irradiation at initial pH values in

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Table 3. Visible Light-Driven H_2 Production with *Db* [NiFeSe]-H Attached on RuP-Sensitized TiO_2 Nanoparticles in TEOA Buffer under Anaerobic Conditions[a]

	TOF[b] $\pm$ σ/s^{-1}	H_2[b] $\pm$ σ/μmol H_2 h^{-1}	H_2 (4 h)[b] $\pm$ σ/%
standard conditions[a]	50 $\pm$ 3	3.56 $\pm$ 0.17	6.69 $\pm$ 0.56
RuP Dependence			
0.02 μmol of RuP	48 $\pm$ 9	3.42 $\pm$ 0.62	6.07 $\pm$ 1.05
0.5 μmol of RuP[c]	55 $\pm$ 6	3.96 $\pm$ 0.46	7.87 $\pm$ 0.84
Db [NiFeSe] Dep.			
0.2 μM	49 $\pm$ 7	0.71 $\pm$ 0.08	1.27 $\pm$ 0.16
5 μM	17 $\pm$ 1	6.27 $\pm$ 0.53	10.87 $\pm$ 0.12
100 μM	1.9 $\pm$ 0.2	14.59 $\pm$ 1.1	18.86 $\pm$ 0.30
TiO_2 Dependence			
1 mg (0.02 μmol of RuP)	24 $\pm$ 8	1.74 $\pm$ 0.76	2.74 $\pm$ 1.19
25 mg (0.5 μmol of RuP)	58 $\pm$ 16	4.23 $\pm$ 1.22	7.72 $\pm$ 0.72
pH Dependence			
pH 4	18 $\pm$ 3	1.29 $\pm$ 0.24	2.13 $\pm$ 0.57
pH 5	37 $\pm$ 1	2.64 $\pm$ 0.06	5.36 $\pm$ 0.47
pH 6	54 $\pm$ 6	3.88 $\pm$ 0.42	7.24 $\pm$ 0.68
pH 8	36 $\pm$ 5	2.57 $\pm$ 0.26	4.87 $\pm$ 0.66
pH 9	4 $\pm$ 2	0.29 $\pm$ 0.06	0.46 $\pm$ 0.24
Temperature Dep.			
5 °C	9 $\pm$ 2	0.70 $\pm$ 0.18	1.44 $\pm$ 0.20
45 °C	72 $\pm$ 5	5.17 $\pm$ 0.39	7.95 $\pm$ 0.96
Inhibition			
0.5 μmol of RuP[d]	5 $\pm$ 2	0.39 $\pm$ 0.14	0.54 $\pm$ 0.22
+ phosphate (25 mM)	2 $\pm$ 1	0.18 $\pm$ 0.08	0.18 $\pm$ 0.01
Soluble Redox Mediator			
$+MV^{2+}$	40 $\pm$ 3	2.84 $\pm$ 0.25	5.52 $\pm$ 0.30
$+MV^{2+}$, no TiO_2	6 $\pm$ 1	0.44 $\pm$ 0.06	0.85 $\pm$ 0.20
Control Experiments			
no RuP		0.16 $\pm$ 0.02	0.24 $\pm$ 0.02
no *Db* [NiFeSe]-H		0.07 $\pm$ 0.01	0.13 $\pm$ 0.01
no TiO_2		0	0

[a] Standard conditions were employed, unless otherwise noted in the table: *Db* [NiFeSe]-H (20 μL of 1 μM solution) added to dispersion of RuP (0.1 μmol) preattached on TiO_2 (5 mg) in TEOA (25 mM, 4.5 mL) buffer at pH 7 and 25 °C, followed by irradiation with a tungsten halogen lamp (250 W, λ > 420 nm) under anaerobic conditions. [b] Turnover frequency (TOF = molecules of H_2 produced per second and per molecule hydrogenase), H_2 production rate (μmol H_2 h^{-1}) based on the first hour of irradiation, and accumulated H_2 (in reactor vessel headspace, in percent) after 4 h ($\pm\sigma$, standard deviation). [c] RuP was added to the TiO_2 dispersion after *Db* [NiFeSe]-H. [d] *Db* [NiFeSe]-H was added to RuP-sensitized TiO_2.

the range 6−8 (<+0.2 after 4 h), and more significantly at pH 5 (<+0.5) due to the poor buffer capacity under more acidic conditions. Importantly, the particles showed high activity around neutral pH (pH 5−8), the conditions found in the most useful fresh and seawater resources.

The temperature dependence of the photocatalytic activity of *Db* [NiFeSe]-H attached on RuP-sensitized TiO_2 was also tested under standard conditions. Photocatalysis is slow at 5 °C with a turnover frequency of 10 $\pm$ 3 s^{-1} but increases to 72 $\pm$ 5 s^{-1} at 45 °C, reflecting the expectation for the enzyme (which is activity-limiting) to display faster rates at the higher temperature (Supporting Information). The high and relatively constant activity of the particles at 45 °C also demonstrates the excellent hours-long electroactive stability of the enzyme on TiO_2 at elevated temperatures.

This system also exhibited considerable long-term stability in the absence of irradiation. Approximately 65% and 35% of initial activity remained when keeping the particles undisturbed in TEOA buffer, under anaerobic conditions, at room temperature, for either 1 week or 1 month, respectively. The fully assembled particles could be stored in the dark at −80 °C under air, after centrifugation and removal of the supernatant buffer. These particles remained approximately 80% and 50% active after 1 week and 3 months, respectively, after resuspension in TEOA buffer. Replacement of the supernatant solution with enzyme and photosensitizer-free buffer solution confirmed that the particles, not the solution components, are responsible for photocatalysis.

We also investigated if and how the hybrid enzyme-modified nanoparticles would perform under sunlight illumination. During a sunny spring day in Oxford, UK, a H_2 evolution rate of 4.5 μmol H_2 h^{-1} was determined at 35 °C when the system was placed in direct sunlight. The rate of production of H_2 decreased to about 1.7 μmol h^{-1} (50% of standard conditions) the following day, when the temperature dropped to 20−25 °C and a cloudy sky decreased the sunlight intensity.

This optimized, under standard conditions, visible light-driven enzyme−nanoparticle system (TOF = 50 s^{-1}; 2070 μmol H_2 h^{-1} (mg enzyme)$^{-1}$; 712 μmol H_2 h^{-1} (g TiO_2)$^{-1}$) compares favorably with some previously reported enzymatic, small molecule, and noble metal-based light-driven H_2 production systems on a per active site basis. UV-band gap irradiation of hydrogenase−TiO_2 dispersions in the presence of a sacrificial electron donor around neutral pH has been reported to yield TOFs of up to 0.1 s^{-1} (6 μmol H_2 h^{-1} (mg enzyme)$^{-1}$) in the absence and 1 s^{-1} (70 μmol H_2 h^{-1} (mg enzyme)$^{-1}$) in the presence of methyl viologen with Ca^{2+} as coadsorbate.[26,40,55] Small molecule catalysts, such as cobaloxime complexes, catalyze H_2 production either electrocatalytically or photocatalytically by visible light in MeCN/H_2O mixtures in the presence of a Pt chromophore and TEOA, giving TOF values up to 0.06

s^{-1} at pH 8.5.[56,57] Visible light irradiated RuP-sensitized TiO_2 (P-25) particles loaded with Pt (3% by mass) in a pH 3.0 EDTA-buffered solution produced up to 10 600 μmol H_2 h^{-1} (g TiO_2)$^{-1}$. Although a direct comparison with our system is difficult considering the different amounts of H_2 production catalyst attached to TiO_2, the high Pt loading (0.77 μmol of Pt per 5 mg of TiO_2) results in a 15 times higher activity (per gram TiO_2) than the one shown by our standard system, but on a per catalytic unit basis this amounts to a 10^3 fold lower TOF (0.04 s^{-1}, using a pair of Pt atoms as the minimum functional unit).[44]

Conclusions

A wide variety of hydrogenases and photosensitizers co-attached to a TiO_2 nanoparticle have been investigated for their ability to function in sunlight-driven H_2 production. We have identified a specially suited, titaniaphilic, hydrogenase, *Db* [NiFeSe]-H, which not only binds strongly to TiO_2, but also sustains a high electrocatalytic activity under easy-to-adopt conditions, an important requirement for photocatalytic H_2 production. In an optimized system, this enzyme is very stable (even after prolonged exposure to air) with a turnover frequency of 50 (mol H_2) s^{-1} (mol enzyme)$^{-1}$ upon visible light irradiation, when attached to a RuP-sensitized TiO_2 at pH 7 and 25 °C, using TEOA as electron donor. It thus provides a benchmark and reference on a per active site basis for future systems. Although this rate is lower than the maximum rate that hydrogenases can acquire, it is remarkably high considering the small driving force acting on the enzyme to produce H_2 during irradiation. This direct-electron transfer controlled rate is 6 times higher than the one obtained by bimolecular (and probably) diffusion-controlled photo H_2 production when replacing TiO_2 by MV^{2+}. Our results show that, despite its large size and the complexity of its internal electron-transport system, *Db* [NiFeSe]-H is an excellent catalyst for artificial photosynthetic systems and proves the capability for achieving very efficient sunlight conversion without precious metals.

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Supporting Information Available: UV−vis data from dye-adsorption studies (Figures S1−S3), pictures of the photo H_2 system before and after irradiation and using different amounts of TiO_2 (Figure S4), time-dependent H_2 production profiles at various pH values (Figure S5), various temperatures (Figure S6), amounts of RuP-sensitized TiO_2 (Figure S7), amounts of *Db* [NiFeSe]-H (Figure S8), and exposure of the photo H_2 system to air and light (Figure S9). This material is available free of charge via the Internet at http://pubs.acs.org.

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